Calcium Ion Gradients and Dynamics in Cultured Skin Slices of Rat Hindpaw in Response to Stimulation with ATP

Moe Tsutsumi[1], Sumiko Denda[1], Kaori Inoue[1], Kazuyuki Ikeyama[1] and Mitsuhiro Denda[1]

Ionotropic receptors, originally found in the brain, were recently also identified in epidermal keratinocytes. Moreover, concentration gradients and movement of calcium are crucial in epidermal homeostasis. Thus, imaging of calcium in the living epidermis is expected to provide insight into epidermal physiology and pathology. Here we describe the imaging of calcium dynamics in the living epidermis of cultured skin slices. The basal calcium concentration was highest in the upper layer of the epidermis. The increase of intracellular calcium in response to adinosine triphosphate (ATP) varied in each layer of epidermis, and was greater at the bottom than in the uppermost layer. Further, the extent of elevation of intracellular calcium in response to ATP in cultured keratinocytes varied depending on the level of differentiation. These results suggest that the response to stimulation of keratinocytes in cultured skin slices varies depending upon the location (depth) within the epidermis.

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INTRODUCTION

The distribution and dynamics of calcium ions are crucial in epidermal homeostasis and signal transmission. There is a marked calcium concentration gradient in healthy epidermis, but this disappears immediately when the stratum corneum barrier is disrupted (Mauro *et al.*, 1998; Denda *et al.*, 2000). Recovery of the barrier function and recovery of the calcium gradient are both blocked by occlusion of skin with a water-impermeable membrane (Menon *et al.*, 1994). It has also been shown that the calcium gradient is strongly associated with keratinocyte differentiation (Elias *et al.*, 2002). Thus, localization of calcium appears to be important for both barrier homeostasis and terminal differentiation of epidermis. However, epidermal calcium dynamics has not been studied in intact skin.

Calcium dynamics may be important in perception at the skin surface. Peripheral nerve fibers had long been believed to be at the forefront of such perception, but we have shown that several pain receptors are expressed in epidermal keratinocytes (Denda *et al.*, 2001b, 2002), and we have suggested that epidermal keratinocytes may be involved in perception (Denda *et al.*, 2007a). This concept is supported by the recent cloning of a series of receptors, which are activated by temperature, mechanical stress, osmotic pressure, and chemical stimuli (Dhaka *et al.*, 2006). Many of these receptors are expressed in epidermal keratinocytes (Denda *et al.*, 2007a). As regards the signaling pathway from these receptors, Koizumi *et al.* (2004) demonstrated that mechanical stimulation of a single keratinocyte induces intracellular calcium elevation not only in that cell, but also in its neighbors, and this response can be prevented by application of apyrase, an adinosine triphosphate (ATP) breaker. They also studied a co-culture system of keratinocytes and neurons, and found that mechanical stimulation of a single keratinocyte induces excitation of neurons. This excitation is also blocked by apyrase (Koizumi *et al.*, 2004). Thus, mechanical stress applied to a single keratinocyte can be signaled to surrounding cells by ATP, and can also evoke excitation of the peripheral nervous system. We recently demonstrated that air-exposed keratinocytes release ATP, which induces intracellular calcium propagation in cultured human keratinocytes (Denda and Denda, 2007). The mechanism has not been clarified. We also demonstrated that TRPV4, which is activated by osmotic pressure, is involved in epidermal barrier homeostasis (Denda *et al.*, 2007b). TRPV4 might be a sensor of air exposure, leading to ATP release (Denda *et al.*, 2007a). We found that the propagation of intracellular calcium is blocked by an ATP receptor blocker (Denda and Denda, 2007), so ATP might be important in signal transduction in response to both mechanical and osmotic stress.

Purinergic receptors (P2) are crucial in signal transduction by ATP in the peripheral and central nervous system (Burnstock and Williams, 2000). For example, P2X3 is a calcium channel receptor, which is associated with pain induced by inflammation (Cockayne *et al.*, 2000). We have

[1]*Shiseido Research Center, Shiseido Co., Ltd., Yokohama, Japan*

Correspondence: Dr Mitsuhiro Denda, Shiseido Research Center, 2-12-1, Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan.
E-mail: mitsuhiro.denda@to.shiseido.co.jp

Abbreviations: ATP, adinosine triphosphate

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demonstrated that the P2X3 receptor is expressed in epidermal keratinocytes, and is associated with epidermal barrier homeostasis (Denda *et al.*, 2002). Not only P2X3, but also various other purinergic receptors are expressed in epidermal keratinocytes, and the distribution of each receptor is different in each layer of epidermis (Inoue *et al.*, 2005). Thus, the response to ATP might be different in each layer of epidermis.

Many studies have employed electrochemical imaging of brain slices to study brain function (Lynch, 1980; Miesenboeck and Kevrekidis, 2005). Here, we present a previously unknown application of this method to observe calcium ion gradients in skin slices from rat hindpaw. We found that keratinocytes in each layer of the epidermis showed distinct characteristics of response to ATP.

RESULTS

Figure 1 shows images of hematoxylin and eosin (a) and nuclear (b) staining of the rat paw skin. The stratum corneum was thick, and the living layer of the epidermis was thicker than in skin from other areas of rats (data not shown).

We next examined the localization of intracellular calcium in cultured skin slices (Figure 2). A steep calcium gradient was observed in the uppermost area of epidermis, as has previously been found in fixed tissue (a) (Mauro *et al.*, 1998; Denda *et al.*, 2000). We repeated this experiment six times. When we observed the calcium gradients, we also evaluated how much stratum corneum had been removed, based on the thickness. In five cases out of six, approximately half of the stratum corneum had been removed. At 2 hours after removal of the stratum corneum, high levels of calcium were observed in the deeper areas (b). (A period of 2 hours was required for preparation of the skin slices and incubation with the calcium indicator.) Moreover, white particles were observed at the basal layer. These might represent the localization of calcium in limited areas (b, arrows). These particles were observed in several sections, but we cannot yet rule out the possibility that they might be artifact. To confirm the viability of the tissue, we employed a probe that forms an impermeable fluorescent product in living cells (CellTracker; Molecular Probes). This probe reacts with glutathione in the cells to form a fluorescent dye. In cultured cells, glutathione and glutathione transferase are ubiquitous, so the probe indicates cell viability (Lantz *et al.*, 2001). As shown in Figure 2c, fluorescence was observed throughout the epidermis, that is, cells in the slice were alive during the observation.

Then, we applied ATP to the skin slices and observed the dynamics of intracellular calcium (Figure 3). Slices were put into a continuous perfusion chamber and $[Ca^{2+}]i$ was measured by means of the fura-2 method. The phase contrast image of the slice before ATP application is shown in Figure 3a. The living layer of the epidermis is located between the white arrows. The time course of intracellular calcium concentration after ATP application at the region indicated by the white outlined square in Figure 3a, is shown in Figure 3b as false-color ratiometric images (F340/F380). ATP was applied at 2 minutes and 50 seconds after the beginning of the observation. White numbers on the upper right of each figure indicate the time in seconds after the application of ATP. ATP exposure induced an increase of epidermal $[Ca^{2+}]i$ in the skin slices, and $[Ca^{2+}]i$ subsequently recovered to baseline. Wavefronts commonly originated in or near the basal layer when the slices were exposed to ATP, and propagated to the

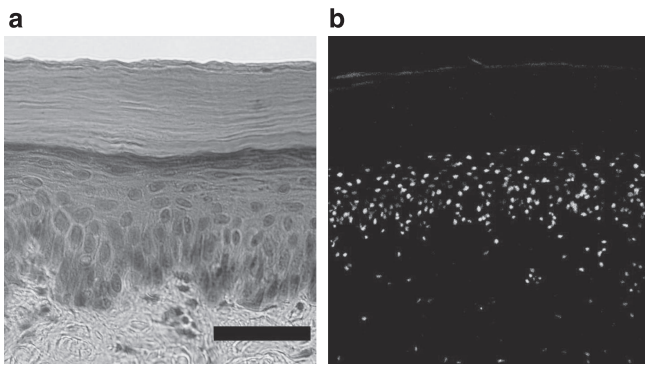

Figure 1. Hematoxylin and eosin (a) and nuclear (b) staining image of the rat paw skin. Thick stratum corneum was observed (**a**). (**a**) Hematoxylin and eosin staining was carried out on fixed sections and (**b**) nuclear staining on unfixed sections. Bar = 50 μm.

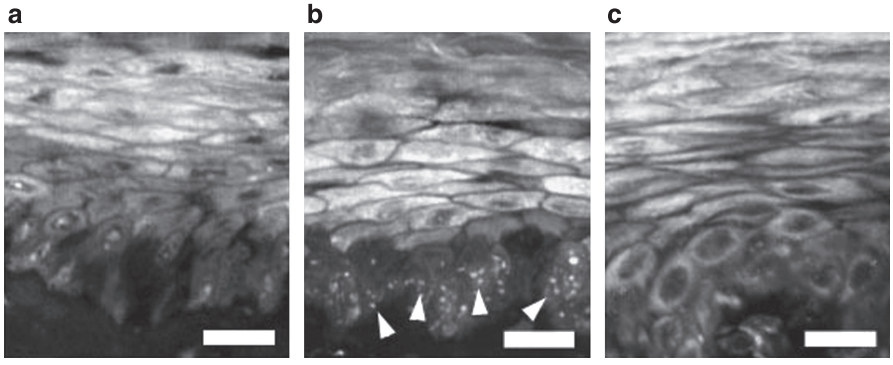

Figure 2. Calcium gradient in cultured slice of rat hindpaw skin. (**a**) High calcium concentration was observed in the uppermost area of the epidermis. (**b**) This shifted to the deeper area, 2 hours after removal of about half of the stratum corneum with cyanoacrylate. White particles were observed at the basal layer (arrows). (**c**) Fluorescence image of the skin obtained with CellTracker. Fluorescence was observed from the bottom to the upper region, i.e., the cells were viable throughout. Bar = 20 μm.

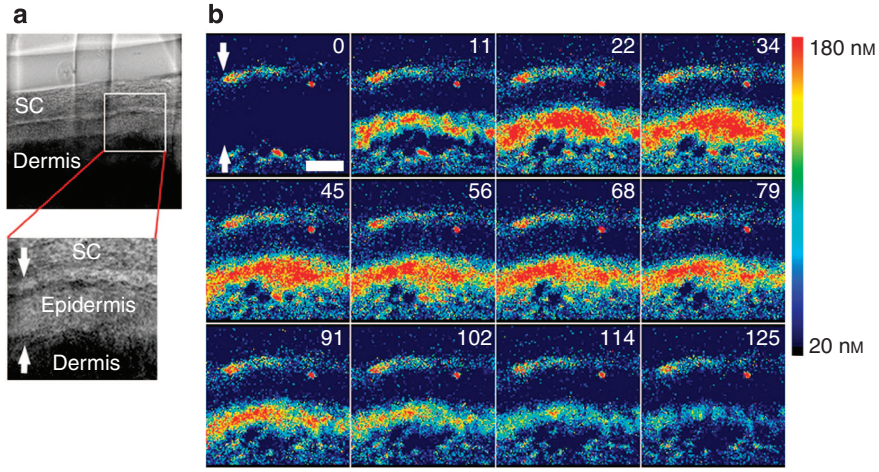

Figure 3. Dynamics of intracellular calcium induced by ATP in rat hindpaw skin. (**a**) A phase contrast image of the test sample. The living layer of the epidermis is located between the white arrows. The time course of change in intracellular calcium concentration after ATP application in the region indicated by a white outlined square in (**a**), is shown in (**b**). The ratiometric images (F340/F380) are shown in false color, where blue, green, yellow, and red indicate increasing intracellular calcium concentration in this order (see scale bar on the right). Bar = 60 μm. ATP was applied at 2 minutes and 50 seconds after the beginning of the observation. White numbers on the upper right of each figure indicate the time in seconds after the application of ATP.

granular layer. We applied ATP to the section from various directions and obtained similar results.

Profiles of $[Ca^{2+}]i$ in different layers of the epidermis are shown in Figure 4. The small inset in Figure 4a shows the points of measurement. The living layer is located between the white arrows. From the superficial layer to the bottom of the epidermis, four areas were chosen (1, blue circle; 2, green; 3, yellow; and 4, red). Each number and color is matched to the profiles of the main graph of Figure 4a. Before the ATP application, the baseline $[Ca^{2+}]i$ was higher at the surface (1, blue) and lower at the bottom (4, red). Upon ATP application, $[Ca^{2+}]i$ immediately started to increase and then recovered to the original level. The extent of the increase was higher at the bottom (4, red) and lower at the surface (1, blue). The profile of each layer is shown in Figure 4b. The elevation of $[Ca^{2+}]i$ after ATP application was greatest in the bottom and least in the top layer.

The results of statistical analysis are shown in Figure 5. Levels of $[Ca^{2+}]i$ in different layer before the application of ATP are shown in Figure 5a. The level in the stratum granulosum was significantly higher than in other layers. We took one slice from each of four different animals and applied 100 μM ATP for 60 seconds. The ratio of elevation of F340/F380 to the initial level was calculated. The ratio in the basal layer was significantly higher than in other layers (Figure 5b).

Then, we evaluated the changes of $[Ca^{2+}]i$ in cultured human keratinocytes. Representative results are shown in Figure 6. Figure 6a shows the profile of proliferating cells and Figure 6b shows that of differentiated cells. $[Ca^{2+}]i$ was elevated following a 20-second application of 100 μM ATP in both cases. However, the extent of the increase was higher in proliferating cells than in differentiated cells. As shown in Figure 6c, there was a statistically significant difference between the two types of cells.

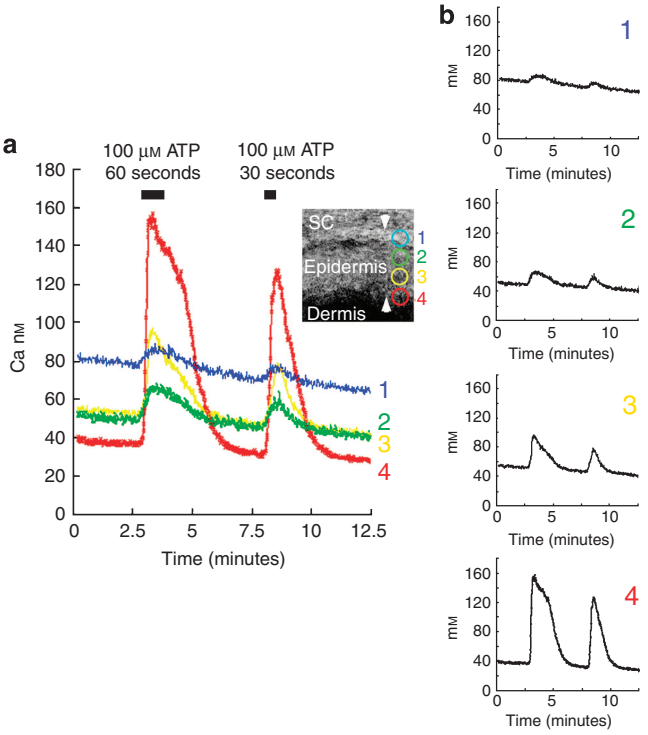

Figure 4. Profiles of intracellular calcium in each layer of the epidermis from rat hindpaw skin after application of ATP. We applied ATP twice at the times indicated in the main figure. The small inset in (**a**) shows the points of measurement. The living layer of the epidermis is located between the white arrows. From the superficial layer to the bottom of the epidermis, four areas were chosen (1, blue circle; 2, green; 3, yellow; and 4, red). Each number and color is matched to the profiles of the main graph of (**a**). Before the ATP application, the baseline $[Ca^{2+}]i$ was higher at the surface (1, blue) and lower at the bottom (4, red). Immediately after the ATP application, $[Ca^{2+}]i$ started to increase and then recovered to the original level. The degree of the increase was higher in the bottom (4, red) and lower in the surface (1, blue). (**b**) The profile of each layer is shown.

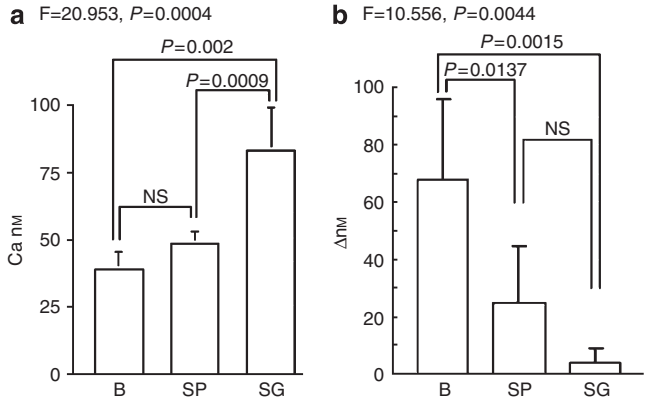

Figure 5. Levels of elevation of intracellular calcium in different layers of cultured skin slices. (**a**) Levels of $[Ca^{2+}]i$ in different layer before the application of ATP are shown. (**b**) Levels of $[Ca^{2+}]i$ elevation after the application of ATP are shown. B, basal layer; SP, spinous layer; SG, stratum granulosum. One slice from each of four different animals was used for this experiment ($n=4$). Before ATP application, $[Ca^{2+}]i$ in stratum granulosum was significantly higher than in the other layers. The level of the elevation after ATP application was significantly higher at the basal layer than in the other layers.

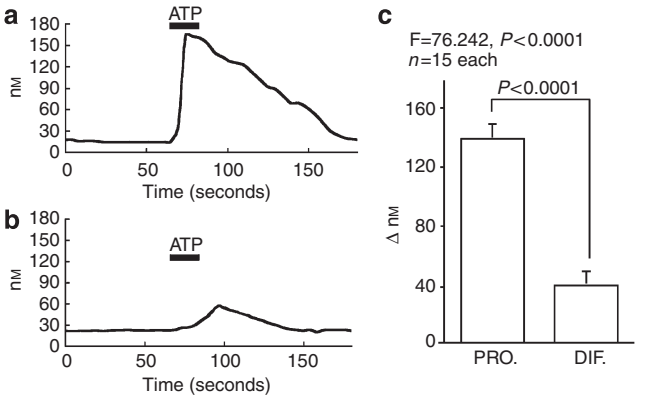

Figure 6. Profile of intracellular calcium of cultured keratinocytes. The profile of proliferating cells is shown in (**a**), and that of differentiated cells is shown in (**b**). Elevation of $[Ca^{2+}]i$ was observed following a 20-second application of 100 μM ATP under both conditions. The results of statistical analysis are shown in (**c**). There was a significant difference between the two types of cells. PRO, proliferating cells; DIF, differentiated calls.

DISCUSSION

In this work, we have succeeded in imaging the calcium gradient in cultured skin slices of rat (Figure 2a), demonstrating the presence of higher basal levels of calcium in the deeper layers (Figure 2b). Previous studies have shown that a steep calcium gradient exists in the uppermost epidermal layer, and this gradient disappears after barrier disruption (Mauro *et al.*, 1998; Denda *et al.*, 2000). The presence of a calcium gradient in rat skin has also been described (Elias *et al.*, 1998), though there is no previous report on calcium distribution in the epidermis of rat paw. Interestingly, we found that the levels of intracellular calcium in individual keratinocytes at the bottom of the epidermis were not homogeneous, though it is possible that this might be an artifact due to cell damage during the process of skin sectioning. After the partial removal of the stratum corneum, white particles were consistently observed in the basal layer (Figure 2b, arrows). Rhod-2, the calcium indicator that we used, can penetrate into the endoplasmic reticulum, so these particles might represent sites of elevated calcium in endoplasmic reticulum, but again we cannot rule out an artifact.

We next investigated the changes of intracellular calcium in response to ATP, because environmental stimuli, such as barrier disruption or exposure to dryness, induce ATP secretion from keratinocytes (Denda *et al.*, 2002; Denda and Denda, 2007). Although the basal calcium level was higher in the uppermost layer of the epidermis than in other layers, the extent of calcium increase in response to ATP was relatively weak in the uppermost layer. Conversely, the basal calcium level was lower in the lower layers, but the response to ATP was higher (Figures 3 and 4). It can be seen that although the profile of calcium elevation was different in each layer the timings of the elevation and recovery were well synchronized (Figure 4). Harmony of signaling in different layers of the epidermis might be important for epidermal homeostasis of intact skin. We previously demonstrated that influx of calcium into keratinocytes delays barrier repair (Denda *et al.*, 2003), so a drastic elevation of intracellular calcium in the uppermost layer of the epidermis might well have a negative effect on barrier homeostasis. Thus, it seems reasonable that the basal level and extent of increase of intracellular calcium in response to stimulus might be individually regulated in the different layers.

At the superficial layer, a barrier recovery response was induced immediately after barrier disruption (Elias and Feingold, 1992), whereas exposure to dryness induced enhancement of barrier function (Denda *et al.*, 1998b). In both cases, increased DNA synthesis was observed in the bottom layer (Proksch *et al.*, 1991; Denda *et al.*, 1998a). Thus, the response to ATP may depend on the differentiation status. Here, we found that keratinocytes indeed showed different responses to ATP, depending on their degree of differentiation (Figure 5b). This is consistent with the observation that expression of neurotransmitter receptors, such as members of the purinergic receptor family, is influenced by keratinocyte differentiation (Inoue *et al.*, 2005). It has also been suggested that ion pumps in keratinocytes might contribute to the electrophysiological status of the epidermis (Denda *et al.*, 2001a). Therefore, differences in expression of receptors or ion pumps may be involved in the different responses of keratinocytes in different layers of the epidermis.

The rat hindpaw has been used for pain research (Tsuda *et al.*, 2003). Fine nerve endings reach the uppermost epidermis and a dense subepidermal nerve plexus is located at the bottom of the epidermis (Navarro *et al.*, 1995). Conversely, pain receptors, such as TRPV1 or P2X3, are expressed at the surface of the epidermis (Denda *et al.*, 2001b, 2002). TRPV1 is activated by heat and chemical stimuli (Dhaka *et al.*, 2006), and we suggested that TRPV1 expressed in keratinocytes may be involved in the sensory system of skin (Denda *et al.*, 2007a). Exposure of keratino-

cytes to stress induced both elevation of intracellular calcium and ATP release, and nerve cells were excited by the ATP released from keratinocytes (Koizumi *et al.*, 2004). Thus, ATP and the P2 receptor might operate as a signal transmission system from the forefront of epidermis to the peripheral nerve fibers. We demonstrated that ultraviolet radiation induced ATP and IL-6 generation, and that both responses were blocked by a P2 receptor antagonist (Inoue *et al.*, 2007). In this study, marked elevation of intracellular calcium at the bottom layer of the epidermis was observed after ATP application (Figures 3 and 4). Changes of intracellular calcium dynamics by P2 receptors might therefore be related to the inflammatory response to environmental stimuli, such as ultraviolet radiation. It should be noted that not only ATP, but also other neurotransmitter receptor agonists or antagonists can induce changes of intracellular calcium concentration in epidermal keratinocytes (Denda *et al.*, 2002, 2003; Fuziwara *et al.*, 2003). The corresponding receptors might mediate information transfer from epidermal keratinocytes, acting as the interface between the body and environment. Pharmacological intervention in the regulation of calcium dynamics through these receptors may yield new clinical methodology for treating various skin diseases that involve abnormal barrier homeostasis or epidermal proliferation.

The method described here should be helpful to explore the putative information processing system operating in the epidermis. Iijima *et al.* (1996) demonstrated a specific pattern of electrochemical dynamics in the CA1 area of the hippocampus, and Tsien *et al.* (1996) reported that the *N*-methyl-D-aspartate receptor in the CA1 area is crucial in memory and learning. These studies illustrate the value of imaging in tissue slices for studies of information processing.

In conclusion, we observed calcium gradients in cultured slices of living epidermis, as previously seen in frozen (Denda *et al.*, 2000) or fixed skin (Mauro *et al.*, 1998). Moreover, we found that the elevation of intracellular calcium after ATP application was different in each layer of epidermis. The basal level of calcium was higher in the uppermost layer than in the bottom layer, but the extent of the calcium elevation was greater in the bottom layer than in the uppermost layer. Cultured keratinocytes also showed differences in the extent of the ATP-induced calcium elevation, depending on their level of differentiation. These results are consistent with the existence of a heterogeneous and sophisticated system in the epidermis for responding to environmental stimuli.

MATERIALS AND METHODS

Animals

Subjects were 2- to 4-month-old male Wistar rats (Hoshino, Saitama, Japan). Animals were anesthetized with halothane and the paw skin was excised. All experiments were approved by the Animal Research Committee of the Shiseido Research Center in accordance with the National Research Council Guide (1996).

Histological observation

The excised skin from Wistar rat hindpaws was fixed in 10% neutral-buffered formalin for 24 hours at 4 °C, processed through a standard paraffin embedding protocol, and cut into 4- to 5-mm thick sections, which were processed for routine hematoxylin and eosin histochemistry (hematoxylin and eosin staining). For nuclear staining, sliced skin sections were immersed in Hoechst 33258 solution (1:1000; Dojindo, Kumamoto, Japan).

Skin slice preparation and calcium imaging

Vertical, 300 μm thick slices from hindpaw skin were prepared using a vibratome (VT1200 S; Leica Microsystems). Slices were cut in a cutting solution containing 136 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 22 mM $NaHCO_3$, 30 mM glucose, 170 mM sucrose, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$. After slicing, slices were incubated in the cutting solution at room temperature until used for experimentation. We removed approximately half of stratum corneum with cyanoacrylate. We evaluated the level of the damage from histological observation.

For calcium imaging, slices were stained for 1 hour by immersing them in the cutting solution containing fura-2 or Rhod-2 (Molecular Probes; 20 μM in DMSO, <0.1% final concentration). After staining of the slices, imaging was carried out with an inverted epifluorescence microscope (IX70; TS Olympus, Tokyo, Japan), equipped with a 75 W xenon lamp and band-pass filters of 340 and 380 nm for fura-2. The images were captured with a high-sensitivity charge-coupled-device camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan) coupled with a Ca^{2+} analyzing system (AQUACOSMOS/RATIO; Hamamatsu Photonics). Calibration of calcium concentration was carried out with a Fura-2 Calcium Calibration Kit (Molecular Probes).

For the observation of sections stained with Rhod-2, an upright microscope (Eclipse; Nikon, Tokyo, Japan), equipped with CSU-X1 laser-scanning unit set at 561 nm (Yokogawa, Tokyo, Japan) was used. The images were captured with an EM-CCD camera (ImagEM; Hamamatsu Photonics) controlled by Volocity software (Improvision, UK). Recordings were made at room temperature.

For viability measurements with CellTracker, sliced skin sections were immersed for 1 hour in 10 μM CellTracker (Molecular Probes).

Cells and cell culture, and Calcium imaging

Normal human epithelial keratinocytes were purchased from Kurabo (Osaka, Japan). Normal human epithelial keratinocytes were cultured in Humedia-KG2 (Kurabo). The medium was replaced every 2–3 days. Normal human epithelial keratinocytes (passage 1–3 cells) were seeded onto collagen-coated cover glass chambers (Nalge Nunc, Naperville, IL) and used within 5 days. Normal human epithelial keratinocytes were grown to approximately 60–80% confluency (subconfluent), 100–120% confluency (confluent), and 100–120% confluency at 24 hours after treatment with 1.8 mM Ca^{2+} (differentiated). Changes in $[Ca^{2+}]i$ in single cells were measured by the use of fura-2 (Molecular Probes). Cells were loaded with 5 μM fura-2 at 37 °C for 30 minutes. After loading, the cells were rinsed in balanced salt solution containing 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10 mM D-glucose (pH 7.4), and then further incubated for 10 minutes at room temperature to allow deesterification of the loaded dye.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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